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Research Article

Polyploidization Facilitates Biotechnological *In Vitro* Techniques in the Genus *Cucumis*

Dagmar Skálová, Vladan Ondřej, Ivana Doležalová, Božena Navrátilová, and Aleš Lebeda

Department of Botany, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

Correspondence should be addressed to Dagmar Skálová, dagmar.skalova@upol.cz

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Prezygotic interspecific crossability barrier in the genus *Cucumis* is related to the ploidy level of the species (cucumber (*C. sativus*), x = 7; muskmelon (*C. melo*) and wild *Cucumis* species, x = 12). Polyploidization of maternal plants helps hybridization among other *Cucumis* species by overcoming prezygotic genetic barriers. The main objective of this paper is to compare the results of several methods supporting interspecific crosses in cucumber without and with polyploidization (comparison between diploid (2x) and mixoploid (2x/4x) cucumber maternal plants). Mixoploid plants were obtained after *in vivo* and *in vitro* polyploidization by colchicine and oryzalin. Ploidy level was estimated by flow cytometry. Embryo rescue, *in vitro* pollination, and isolation of mesophyll protoplast were tested and compared. Positive effect of polyploidization was observed during all experiments presented by higher regeneration capacity of cultivated mixoploid cucumber embryos, ovules, and protoplasts. Nevertheless, the hybrid character of putative hybrid accessions obtained after cross *in vivo* and *in vitro* pollination was not confirmed.

1. Introduction

Polyploidization (chromosome doubling) can be either natural (spontaneous) or induced. Spontaneous chromosome doubling is due to endomitosis and endoreduplication [1]. Induced polyploidization is routinely used by colchicine treatment; but fewer toxic agents (oryzalin, trifluralin, nitrous oxide, etc.) are now being used [2]. Nevertheless, the colchicine manipulation was still used for chromosome doubling. High concentration and duration of this mitotic toxin was used during the experiments with petiole explants of *Echinacea purpurea* L. [3]. Polyploidization has played a major role in ornamental plant breeding. Wu et al. [4] focused on the polyploidization experiments within the Oriental lilies (diploid oriental cultivars of *Lillium*—"Con. Amore" and "Acapulco") (to obtain diploid eggs). Their results implied that the polyploidization is a powerful method to create novel variations in the Oriental lilies. Induced polyploidization may facilitate to produce hybrids among species containing different basic chromosome numbers [5–7]. Some few preliminary results relating to polyploidization in interspecific hybridization in the genus *Cucumis* have been reported [8]. This step may overcome the prezygotic barrier, caused by different chromosome numbers, and we can produce interspecific hybrids. Hybridization within the genus *Cucumis* has been used in breeding programs. By using several biotechnological methods (embryo rescue, *in vitro* pollination, protoplasts isolation, and fusion), hybrids between *C. sativus* with *C. melo* and other *Cucumis* genotypes were produced in sporadic cases. Chen and Staub [9] restored fertility by chromosome doubling with colchicine in F_1 plants from hybridization between *C. sativus* and *C. hystrix*.

Within the genus *Cucumis*, the basic chromosome number is variable caused by two distant origins. Cucumber (*C. sativus*) with x = 7 is considered to be of Asiatic origin and muskmelon (*C. melo*) and wild *Cucumis* species are of African origin (x = 12). Transfer of economic important genes such as resistances to various pathogens, found in the wild *Cucumis* species, is a successful interspecific hybridization methodology that needs to be developed [10].

Embryo rescue saves the immature embryos after interspecific crossing. Hybrids were obtained between *C. sativus* and other *Cucumis* genotypes [11, 12]. Furthermore, hybrids

Table 1: *Cucumis* species, abbreviations, and accession numbers used in this study.

Cucumis species	Abbreviation	Accession number
C. sativus (SM-6514/line)*	CS	CZ 09H3900768
C. sativus (Stela F1)**	CSS	CZ 09H3900744
C. sativus (Marketer 430)***	CSM	CZ 09H3900121
C. melo (line MR-1)[1,2]	CM1	PI 124111
C. melo var. Charentais[1]	CM2	PI 261778
C. melo PMR 45[2]	CMx	CZ 09H400597
C. melo WMR 29[2]	CMx	CZ 09H400598
C. melo PMR 5[2]	CMx	CZ 09H400599
C. anguria var. *longipes* [1]	CA	PI 249896
C. zeyheri[1]	CZ	PI 364473
C. metuliferus[1]	CME	PI 292190

Explanatory Notes. *Cucumber genotype used for *in vivo* polyploidization and for following *in vivo* pollination; **cucumber genotype used for *in vivo* polyploidization and for following *in vitro* pollination; *** cucumber genotype used for *in vitro* pollination and for following protoplasts isolation; [1]*Cucumis* genotypes used for *in vivo* pollination with mixoploid cucumber; [2]*Cucumis* genotypes used for *in vitro* pollination with mixoploid cucumber.

in other crops species have been produced through embryo rescue method (e.g., Brassicaceae [13, 14], Lilliaceae [15], and *Lens* [16]). The composition of media and the cultivation conditions play an important role in these crosses.

Embryos are rescued in crosses where postfertilization is a problem, and embryo aborts after 18 to 21 days after fertilization. However, an alternative is to pollinate and fertilize eggs *in vitro* in distantly related species. Isolated ovules and pollen grains are cultivated together in the special media. Interspecific and intergeneric hybrids have been obtained in several cases [17–19]. However, in the *Cucumis* species, hybrid plants have not been reported. The highest level of regeneration, achieved, was the callus formation [20, 21], and true hybridity was not established [22].

In addition to classic sexual hybridization, distantly related species can be hybridized by somatic hybridization. In *Cucumis* species, isolation and fusion of protoplasts from *C. sativus* , *C. melo,* and wild *Cucumis* species have been reported [23, 24]. Plants through protoplast fusion have been reported in the genus *Brassica* [25–27] and genus *Solanum* [24, 28, 29].

This paper reports on utilization of polyploidization in *in vitro* biotechnological methods which could be used for cucumber breeding. The main aim of this work is to describe and compare the results of these *in vitro* technique applications in diploid (2x) and mixoploid (2x/4x) cucumber plants (used as maternal plants in hybridization). The experiments of *in vivo* pollination (interspecific), followed by embryo rescue and *in vitro* pollination (intraspecific and interspecific), and protoplasts isolation in the genus *Cucumis* were analyzed and evaluated.

2. Materials and Methods

2.1. Plant Source. Various accessions of *Cucumis* species were used for different *in vitro* techniques (Table 1). Plant materials originated from the vegetable germplasm collection of the Research Institute of Crop Production (Prague), Department of Gene Bank, at Olomouc, Czech Republic (Web site: (http://www.vurv.cz/), part databases, EVIGEZ) and from the USDA-ARS North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA. Plants were cultivated in a glasshouse (25°C/15°C day/night) in the Department of Botany, Palacký University in Olomouc, Olomouc, Czech Republic.

2.2. Polyploidization In Vivo. Cucumber seedlings were treated with colchicine ($C_{22}H_{25}NO_6$) (wetted cotton wool on the growth apex with 5 mM and 50 mM colchicine solutions for 2 h; rootlets were submersed in 0.5% colchicine for 24 h; [8]). These influenced plants were used in *in vivo* cross-pollination and embryo rescue. Oryzalin ($C_{12}H_{18}N_4O_6S$) was applied by wetted cotton wool on the growth apex with 30, 60, 90, and 150 μM for 2 h and by submersion of rootlets with 15 and 30 μM for 24 h. These obtained plants were used in *in vitro* pollination experiments. The ploidy was determined by flow cytometry [8].

2.3. Polyploidization In Vitro. Cucumber embryos were isolated and treated by oryzalin in cultivation medium in Petri dishes (1/2 MS medium). Two concentrations (25 and 45 μM oryzalin) and three different times (8, 24, and 48 h) were examined. The seedlings produced from these treatments were cultivated on OK medium (Table 2) and they were used for protoplasts isolation experiments. The ploidy was determined by flow cytometry.

2.4. Isolation, Staining of Nuclei, and Estimation of Ploidy Level. Flow cytometry was used to estimate ploidy level of *in vivo* or *in vitro* cultivated plants after colchicine and oryzalin (polyploidization) treatments. The procedure has been described by Skálová et al. [8]. Relative fluorescence of the nuclei was measured using a PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a laser.

Table 2: Cultivation media used in this study.

Medium	Composition of media	References
OK*	MS medium + 20 mg/l ascorbic acid, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar	[30]
ON*	MS medium + 1 g/l casein hydrolysate, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 6 g/l agar	[30]
CW*	MS medium + 5% coconut water, 200 mg/l α-glutamine, 0.01 mg/l IBA, 0.01 mg/l BA, 60 g/l sucrose, 6 g/l agar	[30]
GA*	MS medium + 0.3 mg/l GA_3, 0.01 mg/l IBA, 0.01 mg/l BA, 20 g/l sucrose, 8 g/l agar	[30]
YS**	600 mg/l $Ca(NO_3)_2$ x H_2O, 100 mg/l H_3BO_3, 80 g/l sucrose, 8 g/l agar	[21, 31]
CP**	MS medium + 9.5 mg/l glycine, 500 mg/l casein hydrolysate, 40 g/l sucrose, 4 mg/l IAA, 0.5 mg/l KIN, 5 mg/l GA_3, 40 g/l sucrose, 8 g/l agar	[21, 31]
N**	N medium + 500 mg/l casein hydrolysate, 50 g/l sucrose, 8 g/l agar	[21, 31]
MSN***	MS medium + 30 g/l sucrose, 0.5 mg/l NAA, 1 mg/l KIN, 8 g/l agar	[31]

Explanatory Notes. OK, ON, CW, GA, YS, CP, N, and MSN: the abbreviations for media used during the experiments; MS medium [32]; N medium [33]; *media used for embryo rescue after *in vivo* pollination; **media used for *in vitro* pollination; ***medium used for embryogenesis after *in vitro* pollination; IBA: indole-3-butyric acid; BA: benzyladenine; GA_3: gibberellic acid; KIN: kinetin; NAA: α-naphthalene-acetic acid.

Table 3: Summary of cross *in vivo* pollination *C. sativus* (2x; 2x/4x) ×*Cucumis* spp.

Ploidy of plants/abbreviation	No. of IH pollination	No. of obtained fruits	No. of isolated seeds	No. of isolated embryos	No. of regeneration
2x (CS)	80	36	980	420	12
2x/4x (CSC)	111	72	2376	704	33

Explanatory Notes. IH: interspecific hybridization.

2.5. Media for Embryo Rescue Culture after In Vivo Pollination. Four types of media were used for embryo rescue of potential hybrid embryos derived from interspecific hybridization of diploid (2x) and mixoploid (2x/4x) cucumber maternal plants with other *Cucumis* species. Fourteen days after *in vivo* pollination embryos were cultured on various media (medium OK, ON, CW, and GA; [30]; Table 2). All experiments were repeated (ten embryos were cultured per dish repetitively). The details documented in this experiment design were summarized in Table 3. The variability in the system was reflected using standard deviation.

2.6. Media for In Vitro Pollination. Three types of cultivation media were used for *in vitro* fertilization. Diploid (2x) and mixoploid (2x/4x) cucumber ovules were pollinated by cucumber and muskmelon pollen grains (medium YS, CP, and N; [21, 31]; Table 2). Diploid ovules after successful *in vitro* fertilization (inspected using microscope Olympus CK40) were transferred on media for embryo rescue described above. The mixoploid ovules after fertilization were cultivated on medium for generating embryo-derived calluses (medium MSN; [31]; Table 2). All experiments were repeated (ten ovules were cultured per dish repetitively). The details documented in this experiment design were summarized in Table 4. The variability in the system was reflected using standard deviation.

2.7. Protoplast Isolation. Mixoploid (2x/4x) cucumber plants (obtained after *in vitro* polyploidization) were used for mesophyll protoplast isolation. They were isolated according to Navrátilová et al. [34]. Due to high level of contamination in mixoploid isolated protoplasts, the antibiotics were added to enzymatic solution (400 mg/l ampicillin, 100 mg/l chloramphenicol). The regeneration efficiency (the first cell division, microcallus, and callus formation), the viability, and the density of protoplasts were compared. Viability of protoplasts was established using an Olympus fluorescent microscope BX 60, fluorescein diacetate (FDA) stain [35], and a BW filter. Density of protoplasts was determinate by haemocytometer. The variability in the system was reflected using standard deviation.

2.8. Detection of the Putative Hybrids. The DNA of the putative hybrid plants originated from embryo rescue approach (after *in vivo* cross-pollination) and ovules after *in vitro* cross-pollination were isolated by standard CTAB procedure. The PCRs with specific primers for ITS regions (internal transcribed spacers) were performed by using FastStart PCR Master Kit (Roche). The PCR products were checked by agarose electrophoresis, purified using GeneElute PCR Clean up Kit (Sigma), cloned into the pGEMT vector (Promega), and introduced into *E. coli*. Selected bacterial colonies were cultured and used for plasmid DNAs isolation. Isolated plasmid DNA was sequenced and sequences were compared with both muskmelon and cucumber ITS sequences originated from EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Also BLAST 2.0 program was used to find sequence similarity and homology.

Table 4: Summary of *in vitro* pollination of *C. sativus* (2x; 2x/4x) × *Cucumis* spp.

Ploidy of plants/abbreviation	♂	No. of ♀ and ♂ around	No. of successful fertilization	No. of regenerated ovules
2x (CSS)	CSS	1610	570	220
	CMx	610	370	96
2x/4x (CSSO)	CSM	310	155	89
	CSSO	280	230	58
	CMx	780	250	130

3. Results and Discussion

Induced polyploidization through colchicine and oryzalin treatments in cucumber was effective. There were, always, obtained mixoploid plants (2x/4x) in diploid cucumber maternal plants after colchicine pretreatment (5 mM and 50 mM, wetted cotton wool on the growth apex and submersion of rootlets). These plants were used for *in vivo* hybridization experiments. The effect of oryzalin was evaluated to induce mixoploidy at a lower concentration (15, 30, 60, 90, and 150 μM, wetted cotton wool on the growth apex and submersion of rootlets; 25 and 45 μM oryzalin in 1/2 MS medium). Oryzalin is not as toxic as colchicine; in addition, in contrast with oryzalin, colchicine is carcinogenic. The use of less dangerous polyploidization reagents was suggested in other studies [2, 36]. The mixoploid plants, arisen after oryzalin treatment, were used for *in vitro* pollination and for protoplasts isolation. Methods of *in vivo* cross-pollination and embryo rescue with mixoploid cucumber maternal plants were tested for the first time. Firstly, there were processed 80 pollination treatments with diploid (2x) cucumber maternal plants, and further 111 treatments with mixoploid (2x/4x) cucumber maternal plants (as sources of pollen selected *Cucumis* spp. were used, details were specified in Table 1). In diploid plants, thirty six pollinations were successful (45%; in majority cases *C. melo* was the most successful pollinating partner) and 980 seeds and 420 embryos were isolated. In case of mixoploid plants, seventy two pollinations were successful (65%; in majority cases again *C. melo* was the most successful pollinating partner) and 2,376 seeds and 704 embryos were obtained. Figure 1 shows the difference between successful hybridization in *Cucumis* species using diploid and induced mixoploid lines. *Cucumis zeyheri*, *C. metuliferus,* and especially *C. melo* (var. Charentais) were suitable parents, identified, during this study. These species also showed satisfactory results during embryo rescue experiment [30]. Hybridization using mixoploid maternal plants (documented in Figure 3(a)) showed better results compared to diploid as maternal plants because higher number and higher final regeneration level of the hybrid embryos were observed. Only callus formation, as the highest level of regeneration, was achieved during cross-pollination of diploid cucumber and muskmelon (Figure 3(b)). On the other hand, the intact plants were obtained from cross-pollination between mixoploid cucumbers and muskmelon (*C. melo* var. Charentais) (Figure 3(c)). The putative hybridism of these plants was inspected. All the sequenced samples of cloned ITS regions were determined as *C. sativus*. The homology of obtained sequences with *C. sativus* ITS sequence (AJ488213) was between 98 and 99.5%. Only one sequence showed high level of sequence differences, but BLAST analysis revealed a relation of this sequence to *C. sativus* genome. The differences are probably results of rather a large-scale mutagenesis in mixoploids after colchicine treatment than those recorded in the interspecific hybridization. Embryo rescue rate is related with the composition of media. It proves that specific components have positive effect on young putative hybrid embryos. Addition of coconut water, casein hydrolysate, and gibberellic acid facilitates rescue of hybrid embryos [8, 30, 37, 38]. Based on this evidence, we obtained the best cultivation results on medium GA in both cases (diploid and mixoploid cucumber maternal plants). The half of obtained microcalluses from crosses between 2x × 2x plants were grown on medium GA (four microcalluses *C. sativus* × *C. melo*; two microcalluses *C. sativus* × *C. metuliferus*). Sixteen regenerants were obtained in mixoploid × 2x (eight plants and six microcalluses *C. sativus* × *C. melo*; two microcalluses *C. sativus* × *C. metuliferus*).

We also used *in vitro* pollination and fertilization to generate hybrid. *In vitro* pollination and following fertilization methods were tested in other genera (e.g., genus *Cichorium* [17]; family Brassicaceae [19]). In our study, pollen grains and ovules were isolated from diploid (2x) cucumber and cultivated together. The muskmelon pollen grains were also used to pollinate diploid cucumber ovules. For *in vitro* pollination and fertilization of mixoploid cucumber ovules, we used mixoploid cucumber pollen and muskmelon pollen grains again. The results of successful fertilization after these pollination experiments were summarized in Table 3. Figure 2. shows the differences between the number of successful *in vitro* pollination in diploid and mixoploid cucumber ovules. The number of obtained regenerated ovules (green ovules, or callus formation on ovule tissue) was higher in mixoploid maternal plants (57% for diploid cucumber pollen grains; 25% for mixoploid pollen grains; 52% for muskmelon pollen grains), than in diploid maternal cucumber plants (38% for diploid cucumber pollen grains; 26% for muskmelon pollen grains). The highest level of regeneration was microcallus and no intact plants were obtained in both cases—in diploid (Figure 3(d)) and mixoploid cucumber ovules (Figure 3(e)). Thus, hybrid character of microcalluses derived from diploid ovules was not confirmed [22]. Sequences of the cloned ITS region from mixoploid fertilized

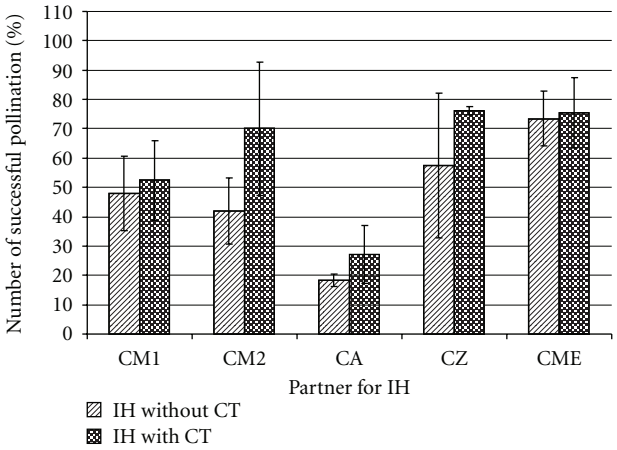

Figure 1: The comparison of successful pollination (% of obtained fruits) in interspecific hybridization between 2x cucumber (IH without CT) and 2x/4x cucumber plants (IH with CT) with other *Cucumis* spp. *Abbreviations:* CM1, CM2, CA, CZ, and CME: set of tested *Cucumis* spp., hybridization partners for cucumber; IH: interspecific hybridization; CT: colchicine treatment; Y-error bars represent standard deviations.

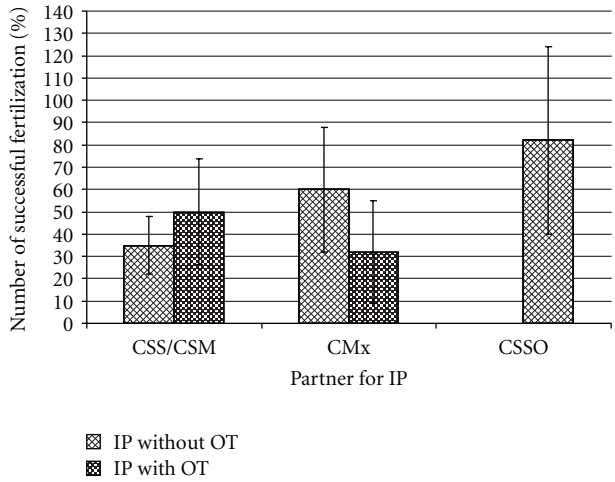

Figure 2: The comparison of successful fertilization (% of growing pollen tubes) after *in vitro* pollination between 2x cucumber (IP without OT) and 2x/4x cucumber plants (IP with OT) with other *Cucumis* species. *Abbreviations:* CSS, CSM, and CMx: set of tested *Cucumis* spp., CSSO: mixoploid *C. sativus* obtained after OT; IP: *in vitro* pollination; OT: oryzalin treatment; Y-error bars represent standard deviations.

ovules showed also nearly 100% homology with ITS region of *C. sativus* (AJ488213) and no clone showed muskmelon ITS.

Naturally, the influence of media composition on successful *in vitro* fertilization was proved. The ovules, which became green, were usually cultivated in medium CP. Especially mixoploid ovules showed the best results on this medium (45% of the regenerated ovules after *in vitro* fertilization with cucumber diploid pollen grains; 49% of regenerated ovules after *in vitro* fertilization with muskmelon pollen grains). Diploid ovules showed the similar results—the expressive regeneration on CP medium (70% of regenerated ovules *in vitro* pollinated by diploid cucumber and muskmelon pollen grains). This medium contains casein hydrolysate, which has also a positive effect on immature embryos developed after *in vivo* pollination. Nevertheless, only callus formation was observed. The media supported embryo rescue (OK, ON, CW, and GA), and the medium for embryo-derived calluses (MSN) did not have positive influence on organogenesis. There was no difference in cultivation of regenerated ovules after transferring them on these media. The microcalluses with size maximally 2 mm were cultivated for twelve weeks without change. The necrosis appeared mostly after the longer cultivation. Popielarska [39] reported only four successful experiments with low percentage of seedlings (2.2-2.3%) that were obtained after self-pollination ovules of *Brassica oleracea* and *Cichorium intybus* and during experiments with sunflower (*Helianthus annuus*).

The protoplasts were obtained from mesophyll tissues of mixoploid cucumber plants produced after *in vitro* polyploidization. The flow cytometry results showed that the ploidy of protoplasts was 2x/4x/8x. Thirty five isolations were performed for mixoploid cucumber accessions; 37% isolations and following cultivations of 2x/4x/8x protoplasts were successful (protoplasts showed the regeneration efficiency, the first cell division (Figure 3(f)), and the microcallus and callus formation (Figure 3(g))). Gajdová et al. [23] recorded an average of 25% regeneration efficiency of diploid cucumber protoplasts. Therefore, the mixoploid cucumber mesophyll protoplasts reached a higher regeneration capacity than diploid protoplasts. The determined average of mixoploid protoplast viability was 75% ($\pm$14%) (Figure 3(h)) and the average density per 1 g of mesophyll tissue was 3.7×10^6 ($\pm 1.9 \times 10^6$). Navrátilová et al. [40] found 86.09% cucumber protoplast viability and the average density 4.36×10^6 per 1 g of mesophyll diploid cucumber tissue. Nevertheless, Gajdová et al. [41] also summarized the average yields of mesophyll protoplasts for different diploid cucumber genotypes (the averages from 1.98×10^6 to 11.85×10^6 per 1 g of mesophyll tissue). The results obtained from experiments with mixoploid protoplasts will be helpful in protoplasts interspecific fusion (somatic hybridization).

4. Conclusions

The polyploidization pretreatments employed for generating interspecific hybridization used in this study are reported for the first time in the genus *Cucumis*. Mixoploid (2x/4x) plants were obtained after colchicine and oryzalin treatment. Several *in vitro* techniques have been utilized for facilitating interspecific hybridization (embryo rescue, *in vitro* pollination, and protoplasts isolation) and the results concerning the usage of diploid and mixoploid cucumber maternal plants were analysed and compared. The positive influence of this procedure was proved especially for *in vivo* cross-pollination between cucumber and other *Cucumis* species.

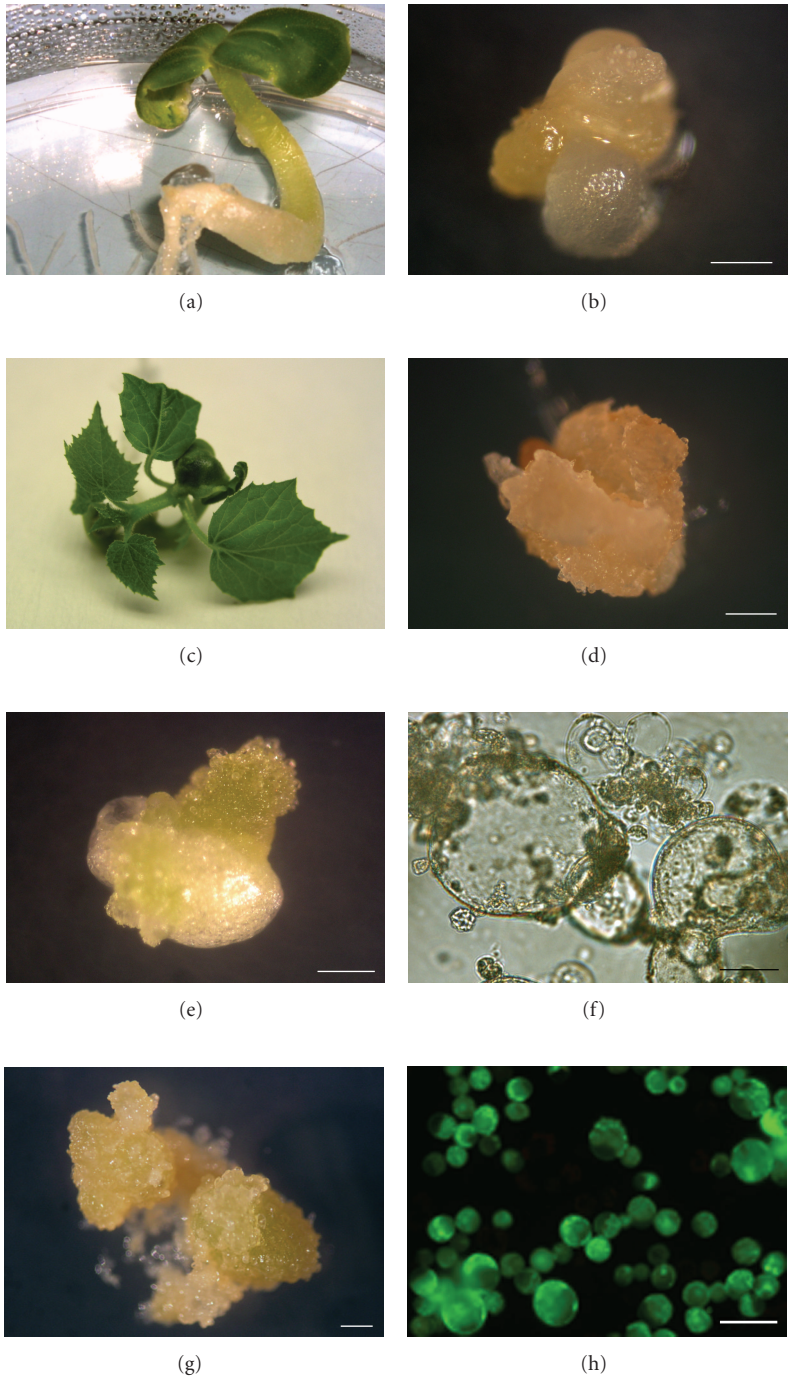

Figure 3: (a) Cucumber mixoploid (2x/4x) seedling after *in vitro* polyploidization by oryzalin. (b) *C. sativus* (2x) × *C. melo* microcallus after *in vivo* pollination. (c) *C. sativus* (2x/4x) × *C. melo* plant after *in vivo* pollination. (d) *C. sativus* (2x) × *C. melo* microcallus after *in vitro* pollination. (e) *C. sativus* (2x/4x) × *C. melo* microcallus after *in vitro* pollination. (f) Differentiation of cucumber mixoploid protoplasts. (g) Microcallus of cucumber mixoploid protoplasts. (h) The viability of protoplasts isolated from cucumber mixoploid mesophyll after FDA staining. Bars in (b, c, e, g): 500 μm and in (g, f): 100 μm.

The intact plants were obtained after crossing mixoploid cucumber plants with muskmelon. The mixoploid character of cucumber ovules during *in vitro* pollination and fertilization was demonstrated. However, only calluses were obtained in both cases (in diploid and mixoploid ovules). Mixoploid protoplasts were isolated from mesophyll of plants treated by oryzalin. The average viability and density and regeneration capacity of protoplasts were evaluated suggesting for projected somatic hybridization. In the end, the polyploidization pretreatments substantially facilitated individual *in vitro* techniques, especially regeneration efficiency in mixoploid embryos and ovules.

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